Phylogeny of Chinese *Oreolalax* and the Use of Functional Outgroups to Select Among Multiple Equally Parsimonious Trees

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Abstract. -Re-analysis of a published data set from Xu et al. (1992) reveals that their proposed phylogenetic tree is not the most parsimonious explanation. Ten shorter, most parsimonious solutions are derived from these data. The progressive functional outgroup approach found a single tree from among the ten to be the best explanation of the data. Such an approach may prove to be a powerful method for dealing with multiple equally parsimonious trees.

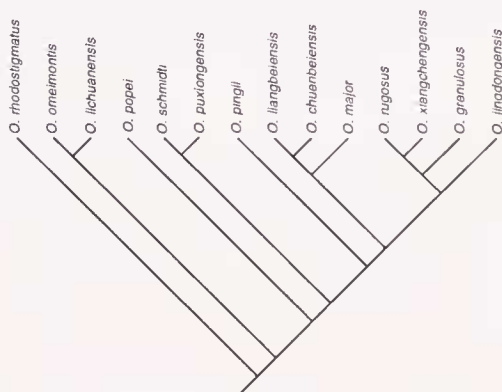*Key words*: Amphibia, Anura, *Oreolalax*, China, Phylogeny, Functional outgroups

Figure 1. Phylogenetic Cladogram of *Oreolalax* proposed by Xu et al. (1992).

Introduction

Although considerable interest has been shown in the evolution of pelobatid frogs relatively recently, the phylogeny of many pelobatids remains largely enigmatic. Xu et al. (1992) proposed a phylogeny for fourteen species of *Oreolalax* (Fig. 1). This genus of pelobatid frogs is endemic to China and most of the 15 described species occur at high elevations (1500 to 3300m). Xu et al. used 29 morphological characters in their analysis (Appendix 1), and these were gathered for the 14 species known at the time of their analysis. Subsequent to the proposed phylogeny, a new species, *O. multipunctatus*, was described (Wu et al., 1993). However, the phylogenetic relationships of this new species were not considered in the description; its position among the other species remained uncertain. Consequently, we undertook a re-examination of the

phylogeny of *Oreolalax* using data provided by Xu et al. (1992) and Wu et al. (1993).

Our initial examination of Xu et al. (1992) showed several problems in their analysis potentially invalidating their conclusions. Because of this, we first pursued a re-evaluation of their data, and subsequently incorporated the new species into our analysis to obtain a more defensible phylogenetic estimation for the genus *Oreolalax*.

Materials and Methods

The data set from Xu at al. (1992) was used in our initial analysis (Appendix II). Our phylogenetic analysis was undertaken using Hennig86 (version 1.5; Farris 1988), Random Cladistics (version 2.0; Siddall 1994) and PAUP (version 3.1.1, Swofford 1993). Branch and bound algorithm (Hendy and Penny 1982) as provided in these software packages was used for all tree searching. The resulting trees were rooted initially by a hypothetical ancestor scored plesiomorphic for all characters based on the hypotheses of Xu et al. (1992). Using Random Cladistics, a permutation tail probability test (PTP; Faith and Cranston 1991) was used for assessing the presence of pattern in the data set, which we assume to represent phylogenetic structure. Bootstrap values (BS; Felsenstein 1985) were used for assessing the relative strength of support for monophyletic groups. Examination of character state distributions on trees was accomplished using MacClade (version 3.04; Maddison and Maddison 1992).

Initial estimations of phylogenetic analysis were accomplished using the unaltered data of Xu et al. (1992). Three methods were exploited for dealing with multiple most parsimonious trees (MPTs): con-
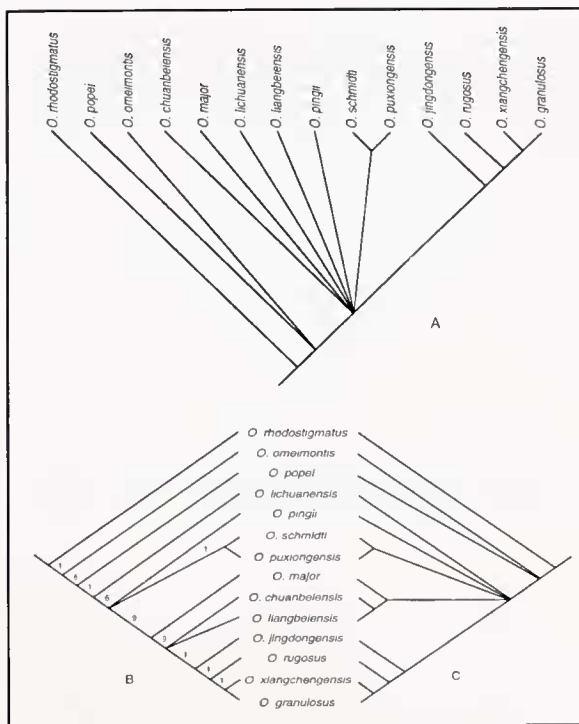
Figure 2. A: Strict consensus tree of 10 MPTs resulting from the original analysis with 7 unresolved nodes. B: 50% majority rule consensus tree with 2 unresolved nodes: 6=0.6; 9=0.9; 1=1.0. C: Adams consensus tree with 4 unresolved nodes.
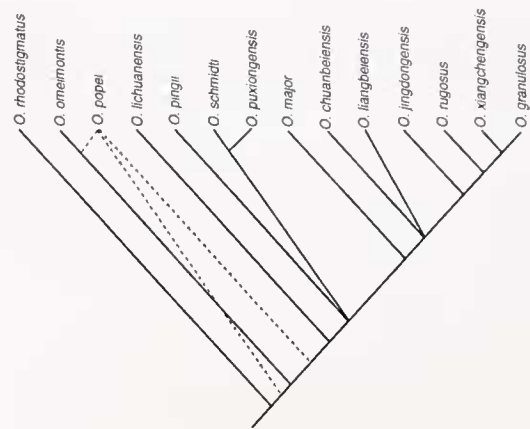

Figure 3. Three MPTs resulting from successive approximations weighting. The unstable position of *O. omeimontis* and *O. popei* is responsible for the three trees.

sensus evaluation, successive approximations weighting (SAW: Farris 1969, 1988), and functional outgroup approach (FOG) modified from Watrous and Wheeler (1981). Following resolution of the tree for the initial 14 species treated by Xu et al., the position of *Oreolalax multipunctatus* relative to the other species was estimated.

Results

Our PTP test showed the data set of Xu et al. has pattern which is significantly different from random. A PTP value of $p < 0.001$ was obtained based on 999 randomized data sets. The minimum length tree derived from the randomized data was 64 steps whereas the minimum length tree from the original data was 58 steps. We interpret this pattern to reflect the presence of phylogenetic structure.

Ten MPTs of 58 steps were found, each with a consistency index (CI; Kluge and Farris 1969) of CI = 0.50, and a retention index (RI; Farris 1989) of RI = 0.59. When the data are mapped onto the tree of Xu et al., their tree was observed to have a length of 61 steps (CI = 0.47; RI = 0.54). Using PAUP, we found no

fewer than 7000 equally or more parsimonious solutions having 61 steps; we terminated the program after 30 minutes analysis.

A strict consensus tree, Adams consensus tree (Adams 1972), and 50% majority consensus tree were generated from the ten MPTs respectively. The strict consensus tree (Fig. 2a) showed low resolving power. Only seven of 13 nodes were resolved demonstrating much conflict among various patterns of relationships. The Adams consensus tree (Fig. 2b) gave a slightly better resolution with four nodes unresolved. Finally, the 50% majority consensus tree (Fig. 2c) left only two nodes unresolved. In all analyses, *O. rhodostigmatus* always appeared at the base of the ten MPTs.

As another means of selecting a preferred tree from among the ten MPTs, we used SAW. Following the successive re-weighting of characters based on the RI, three trees were found. When the character weights were re-set to 1, each tree had a length of 58 steps, and thus all fall within the suite of initial MPTs. Among the three trees, the position of *O. omeimontis* and *O. popei* were unstable, accounting for three alternative trees, and two other nodes were resolved as polytomys (Fig. 3).

We had concern that the plesiomorphie states may not have been correctly identified as no outgroup was used in the analysis, although the ancestral states theoretically represent the conditions observed in *Leptobrachium*. Given this, and the observation that, in general, the closer the outgroup is to the ingroup the more likely the polarization of character states will be accurately determined, we used a functional outgroup (FOG) approach (Watrous and Wheeler 1981) to
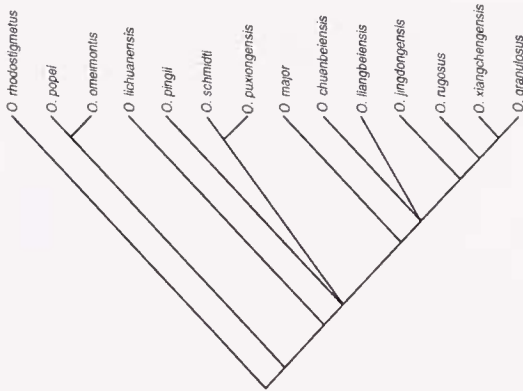
Figure 4. Strict consensus tree of 2 MPTs resulting from using Oreolalax rhodostigmatus as functional outgroup.

Figure 5. Resulting tree using Oreolalax lichuanensis as functional outgroup.

examine the data. Because *O. rhodostigmatus* always appeared at the base of the ten MPTs, this species was used as our FOG. We excluded the hypothetical ancestor from our secondary and subsequent analyses.

Our FOG approach revealed two equally parsimonious trees, each with 53 steps (=58 steps with HA; CI = 0.54, RI = 0.60). Neither tree was the same as resolved from our SAW evaluation but both were among our original ten MPTs. The strict consensus tree shows two unresolved nodes (Fig. 4). For resolving the ambiguous nodes, a closer FOG, *Oreolalax lichuanensis*, was selected. Taxa below the FOG (*O. rhodostigmatus*, *O. popei* and *O. omeimontis*) were excluded from this analysis. A single MPT with 37 steps was found (=58 steps with the deleted taxa; CI = 0.67, RI = 0.70) (. 5). It is one of the ten MPTs from the original analysis. Examination of character state distributions on this tree showed no potential for further resolution to the relationships of *O. chuanbeiensis*, *O. liangbeiensis* and more terminal taxa. Combining the results of our progressive FOG analysis, a single cladogram emerged as our preferred tree (Fig. 6). Thus, our tree is left with one unresolved polytomy.

Bootstrap (BS; Felsenstein 1985; Siddall 1994) was used to assess strength of support of monophyletic groups. The BS value based on 999 randomized data sets was mapped on the resulting tree (Fig. 6). Three nodes were shown to be well supported by BS that have values over 0.5. The *O. schmidti* and *O. puxiongensis* clade has a highest value of 0.840, while the *O. jingdongensis*, *O.rugosus* and *O. granulosus* clade has the second highest value of 0.732. The ingroup member excluding *O. rhodostigmatus* from the clade
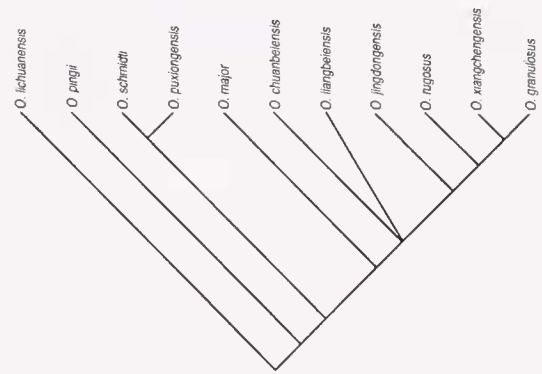
and has a high value of 0.634. The weakest support from BS is the *O. popei* and *O. omeimontis* clade that has a value as low as 0.061.

Finally, the data for *Oreolalax multipunctatus* were combined with those of Xu et al. (1992; Appendix II). From the characters available for all ingroup taxa, *O. multipunctatus* was resolved as either the sister group of *O. schmidti* or *O. puxiongensi*, both of which were most frequently resolved as sister taxa. The missing data were responsible for the ambiguity.

Discussion

Phylogeny of *Oreolalax*

Oreolalax rhodostigmatus was consistently resolved as the sister of all other species in the genus. Three characters (8, 10, 18; Appendix 1) separate it from other species. A BS value of 0.634 was calculated for the node excluding *O. rhodostigmatus*. The *rugosus* group of Xu et al. (1992) also appears to be monophyletic and received a relatively high BS value (=0.732). Zhao and Adler (1993) regarded *O. puxiongensis* as a synonym of *O. schmidti*. Here, the highest BS value observed from the data, BS = 0.840, shows that these two taxa are very similar. Although further detailed work is needed to determine the status of *O. puxiongensis*, because *O. multipunctatus* is resolved as the sister species of either *O. puxiongensis* or *O. schmidti*, synonymization of these latter two taxa would also require synonymization of *O. multipunctatus* if a holophyletic classification is to be maintained. Thus, we recommend recognition of all three species until far more detailed evaluations can be made.
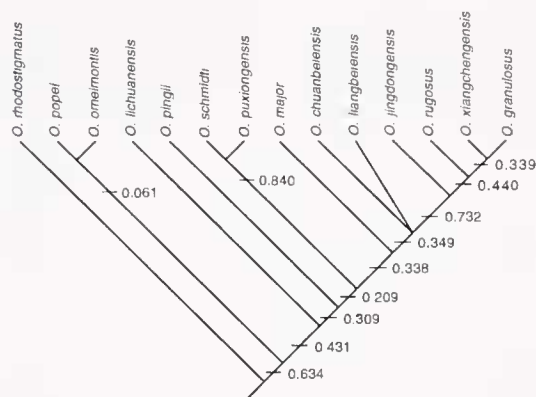
Figure 6. Phylogeny of Oreolalax as resolved by using our progressive functional outgroup analysis. One node remains unresolved. Bootstrap values are provided for each node.

Although *O. popei* and *O. omeimontis* are grouped together on our preferred tree (ure 6), the BS analysis shows a very low value of 0.061. They shared five characters (8, 10, 18, 21, 29). Optimizing these characters on the preferred tree shows that character 8, 10 and 18 are shared by all ingroup members excluding *O. rhodostigmatus*, with 1 or 2 independent reversals. Characters 21 and 29 are shared by all ingroup members with more than 2 independent reversals. These two taxa do not share any uniquely derived characters. We believe that this association should be considered tenuous because of the low BS value, and the resolution of a different set of relationships for these two taxa in our SAW evaluation (Fig. 3). More work is needed to determine the relationships of these species with the others.

The relationships of *O. chuanbeiensis*, *O. liangbeiensis* and the *rugosus* group are unresolved. There are no unambiguous characters to support any resolution of these three taxa. More characters are required for the resolution of this polytomy.

Most species of *Oreolalax* are distributed along the east edge of the Qinghai-Tibet Plateau. *Oreolalax rhodostigmatus* occurs in the far east of the Qinghai-Tibet Plateau and is almost allopatric in distribution from all other species. The *rugosus* group occurs at the highest elevations, 2100-3300m. This suggests that their common ancestor was adapted for the rigors of alpine life. The speciation within the *rugosus* group appears to be closely associated with the uplift of the Qinghai-Tibet Plateau.

Functional Outgroup Method

Our progressive functional outgroup method may prove to be a powerful method for dealing with multiple equally parsimonious trees, especially when levels of homoplasy are relatively great, and the outgroup is so distant from the in-group that mispolarization of the character states is commonplace. When divergences are great, and characters are constrained to only a few possible alternative states, homoplasy may be so rampant that there is very little resolving power to the data when a taxonomic outgroup is used to polarize character states. Selecting a closer sister group, as in the use of FOGs, may prove particularly beneficial for reducing this problem. Thus, the use of FOGs might possibly result in the defensible selection of a single most parsimonious tree when, otherwise, a suite of equally parsimonious trees would be found.

The application of consensus tree methods has not been recommended as a way of selecting among equally parsimonious solutions, although this stance has not been universally accepted (e.g., Wilkinson 1994). Our trial of this approach with the *Oreolalax* data did not demonstrate high resolving power. The strict consensus tree seeks the unambiguous nodes appearing on all MPTs. It often results in a large number of unresolved nodes. A single taxon with ambiguous relationships may cause the whole consensus tree to be a total "bush". The Adams consensus method improves this problem, but in both our case and in others, it still frequently resolves ambiguous nodes. In our case, strict and Adams consensus left 6 and 5 nodes unresolved respectively. Although the majority rule consensus method often gives a more complete solution, the percentage is arbitrary and it is doubtful that all nodes reflect phylogeny.

Successive approximations weighting (Farris 1969) often reduces the number of cladograms to be considered by restricting attention to the most consistent characters, and hence the "best" evidence (Carpenter 1988). Consequently, some ambiguous nodes that are supported by the "best" evidence may get resolved. However, the ambiguous nodes not supported by the suite of consistent characters will remain unresolved. In our trials, SAW resolved three equally parsimonious solutions. The ambiguous position of *O. popei* and *O. omeimontis* is responsible for these three trees. Character 28, which clusters these two taxa together under FOG analysis, was not included in the "best" evidence; It received a weighting value of 0 from the SAW analysis.

In our case, the high resolving power of FOG analysis results from the changing of the character state polarizations. Some symplesiomorphies which

are useless in providing evidence of common ancestry became synapomorphies after re-polarization. However, we believe that the revised data result in a better solution. For example, the polarity of the states of Character 28, changes after using *O. rhodostigmatus* as the FOG. Unlike in our initial analysis, a derived state united *O. popei*, *O. omeimontis* and *O. liangbeiensis*. Similarly, after using *O. lichuanensis* as our FOG, the polarities of the states of Character 24 changed such that a synapomorphy united *O. schmidti* and *O. puxiongensis* with *O. major*.

Evolution of the Characters

Among the 29 characters used by Xu et al., 10 contributed no information to the phylogeny. Characters 7, 11, 12, 22 have autapomorphic states and hence are phylogenetically uninformative. The derived states of characters 1, 13, 20, 23, and 24 were resolved as independent "gains" (parallelism). Character 28 was interpreted to be two independent reversals. Conversely, 19 characters showed clear evolutionary trends when mapped on our preferred tree. However, although characters 2, 3, 4, 5, 6, 8, 9, 10, 14, 15, 16, 18, 19, 21, 26, and 27 evolved from state 0 to 1, characters 17, 25, 29 show a progression from state 1 to 0. These latter three characters are undoubtedly mispolarized by Xu et al. if our tree reflects the genealogical relationships of the taxa.

Acknowledgments

Appendix I

The Characters used by Xu et al (1992)

1. Quadratojugal either long, overlapping with maxilla (O), or short, not overlapping the maxilla.

2. Spoon-like cartilage either present (0), or absent (1).

3. Columella either long (0), or reduced as a small projection (1).

4. Tympanum either present (0), or absent (1).

5. Tympanic annulus either large, diameter>1.9 mm (0), or either small, diameter<1.3 mm, or absent (1).

6. Post-otic projection of squamosal either present (0), or absent (1).

7. Ilium either short (0), or long (1).

8. Body length of males either greater than 65 mm (0), or less then 56 mm (1).

9. Opening of Eustachian tube either large (0), or small (1).

10. Tympanic status either obvious (0), or hidden (1).

11. Dorsal skin either smooth (0), or warty and granular (1).

12. Skin groove along posterior area of the dorsum either absent (0), or present (1).

13. Belly skin either smooth (0), or granular (1).

14. Colour pattern on belly either present (0), or absent (1).

15. Triangular pattern between eyes either absent (0), or present (1).

16. Toes either webbed (0), or either not webbed or only a trace (1).

17. Vocal sac either present (0), or absent (1).

18. Spines in lip margins either absent (0), or present (1).

19. Spinal patches on the chest either small (0), or large (1).

20. Spines on the chest either small (0), or large (1).

21. Nuptial spines on fingers either small (0), or large (1).

22. Spinal patches on the ventral surface at the base of the upper arm either absent (0), or present (1).

23. Spinal patches on the inner side of the lower arm either absent (0), or present (1).

24. Spinal patches on the back of the upper arm either absent (0), or present (1).

25. Linea musculinae either absent (0), or present (1).

26. Labial tooth formula of tadpole either 1:7-7/1:7-7 or more (0), or 1:6-6/1:6-6 or less (1).

27. Papillae of tadpole nostril more than one circle clear (0), or only half circle clear (1).

28. Number of eggs laid either more than 280 (0), or less than 220 (1).

29. Egg pigment. either present (0), or absent (1).

Apendix II

Coded Data set from Xu et al. (1992). A hypothetical ancestor (HA) was added for analysis.

HA	00000 00000 00000 00000 00000 0000
O. popei	10000 00111 00000 01101 10000 0001
O. chuanbeiensis	00001 00111 00010 01100 00010 1011
O. major	00000 00011 00110 01110 00000 1111
O. schmidti	10000 00111 00011 11000 10001 1111
O. omeimontis	00000 00101 00001 10100 10101 0001
O. puxiongensis	00000 00111 00111 11000 11001 1110
O. lichuanensis	00000 00101 00000 01101 10111 1111
O. rhodostigmatus	00000 01000 00000 01000 10001 0011
O. liangbeiensis	00001 00111 00010 01100 00000 1101
O. pingii	00000 00111 11010 01100 10011 1111
O. rugosus	01111 10111 00000 01100 00000 11-0
O. xiangchengensis	01111 00111 00000 01110 00000 1110
O. jingdongensis	01011 10111 00001 01101 10000 1110
O. granulosus	01111 10111 00000 01110 00010 1011

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